Nitrogen Fixation of Nodulation Mutants of Soybean as Affected by Nitrate

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ABSTRACT

It was previously reported that three soybean (Glycine max [L.] Merr.) nodulation mutants (NOD1-3, NOD2-4, and NOD3-7) were partially tolerant to nitrate when nitrate was supplied simultaneously with inoculation at the time of transplanting. The current study evaluated the effect of short-term nitrate treatment on nitrogenase activity (C2H2 reduction per plant and per nodule weight) and on relative abundance of ureides when nitrate application was delayed until plants were 3 weeks old and nodules were fully developed. Nitrogenase activity of the mutants was similar to that of Williams after an initial 3-week growth period, prior to nitrate treatment. Application of 5 millimolar nitrate resulted in greater inhibition of nitrogenase activity in Williams than in the three mutants. NOD1-3 was most tolerant of nitrate among the mutants tested and showed the highest relative abundance of ureides. Although C2H2 reduction activity per plant for NOD1-3 was higher than for Williams in the presence of nitrate, C2H2 reduction activity per gram of nodules was lower for NOD1-3 than for Williams in the presence and absence of nitrate. Compared to Williams, NOD1-3 had higher nodule ureide concentration and had similar glutamine synthetase activity in nodule tissue, indicating its nodules have normal nitrogen assimilation pathways. Nitrate application resulted in ureide accumulation in nodule tissue as well as in all plant parts assayed. Unexpectedly, nitrate treatment also increased the rate of ureide degradative capacity of leaves in both NOD1-3 and Williams. The data confirmed that nitrogenase activity of the selected nodulation mutants was more, but still only partially, tolerant of nitrate compared with the Williams parent.

The mechanism(s) of nitrate inhibition of soybean (Glycine max [L.] Merr.) nodulation and N2 fixation is far from clear (5, 16). The reported selection of nts2 mutants of soybean (1, 2) provided renewed interest in evaluating nodulation response to nitrate. Schuller et al. (13) showed that nodule activity of the nts382 mutant was less affected by nitrate than was the Bragg parent, when nitrate was added to plants with fully developed nodules. The nts382 mutant, however, had a relatively lower C2H2 reduction activity rate than its parent Bragg (13, 14), a difference they attributed to a method error of the closed system of C2H2 reduction assay. The nts382 line also had higher ureide concentration in its nodule tissue (13), likely due to limited growth of the mutant which may have impaired ureide utilization.

Partially nitrate tolerant mutants of soybean (NOD1-3, NOD2-4, and NOD3-7) have also been selected from mutagenized Williams populations, and initial characterization of these mutants has been reported (4). The data indicated many similar characteristics of our mutants to those reported by Carroll et al. (1, 2). However, it is not known whether the nitrogenase activity of our mutants is tolerant to nitrate per se, since in the previous study (4) the effects of nitrate on infection steps and on subsequent nodule function could not be separated due to simultaneous inoculation and nitrate addition. Using 15N analysis it was determined that NOD1-3 fixed more 15N2 than Williams, when grown on urea, and that all three nodulation mutants symbiotically fixed more N2 than wild type when the mutants were grown on nitrate (11). This confirmed the earlier report based on C2H2 reduction assays (4). In the present report, nitrate treatment was delayed until plant roots were well nodulated and capable of relatively high N2 fixation activity. N2 fixation was then assessed by using the in situ C2H2 reduction assay and determining the relative abundance of ureides in plant parts (7). Nodule GS activity and leaf ureide degradative capacity (allantoin metabolism to glyoxylate) were also determined to evaluate possible differences in nitrogen assimilation pathways of the nodulation mutants and Williams. Results reported here confirm that our nodulation mutants are more tolerant than is the Williams parent, but that the mutants are still only partially tolerant to nitrate.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seeds of wild-type Williams soybean (Glycine max [L.] Merr.) and selected nodulation mutants (NOD1-3, NOD2-4, and NOD3-7) were inoculated with a mixed strain commercial preparation of Bradyrhizobium japonicum (Urbana Laboratories, St. Joseph, MO) and germinated in growth chambers in sand with deionized water. A 14-h photoperiod (650 µmol photons m⁻²s⁻¹) at 28°C and a 10-h dark period at 20°C were provided. The plants were transplanted at 7 d into a modified Hoagland nutrient solution (18). The 7-L hydroponic rooting medium included ion exchange resin columns for maintenance of pH at 6.5 (6). The nutrient solutions were supplemented with 1 mM urea for 1 week after transplanting to alleviate the early N stress which occurs if plants are
cultured without N. Plants then received no nitrogen in the nutrient solution until nitrate treatments were initiated 21 d after sowing. Initial assays for C\textsubscript{2}H\textsubscript{2} reduction activity and relative ureide levels were conducted 21 d after sowing (d 0). Plants were then treated with 5 mM nitrate in the nutrient solution for 2 to 7 d, depending on the experiment, and compared with controls maintained on nil nitrate.

**C\textsubscript{2}H\textsubscript{2} Reduction Assay**

*In situ* C\textsubscript{2}H\textsubscript{2} reduction activity was assayed as previously reported (12) except that the volume of nutrient solution was decreased to 5.5 L, 2 d before assay, so that the major portion of the root nodule mass was no longer immersed and could be assayed without disturbing the root systems. Water was maintained at this level during the assay and subsequent growth so that microenvironmental variations were minimized prior to and during the C\textsubscript{2}H\textsubscript{2} reduction assay. After 30 min incubation with C\textsubscript{2}H\textsubscript{2} in the gas phase (1.5 L), samples were taken for analysis of C\textsubscript{2}H\textsubscript{4} formation. *In vivo* C\textsubscript{2}H\textsubscript{2} reduction assays were also conducted by sealing the nodulated root system in gas-tight jars and injecting 10% (v/v) C\textsubscript{2}H\textsubscript{2}. Following a 30 min incubation at 30°C, the samples were analyzed for C\textsubscript{2}H\textsubscript{2} and C\textsubscript{2}H\textsubscript{4}.

**Sample Preparation and Analysis for Relative Abundance of Ureides**

Nodules, stems plus petioles, leaves, and nodulated roots were separated and dried at 60°C for 48 h. The tissues were ground and 100 mg tissue samples were extracted in boiling water for 2 min as described by Herridge (7). Total ureides (allantoin plus allantoic acid) were determined colorimetrically as the phenylhydrazone of glyoxylate (20). Amino N was estimated by the ninhydrin method with asparagine and glutamine as the standard (8). Nitrate-N was determined by the salicylic acid method (3). The relative abundance of ureides was calculated (9) with the following equation:

\[
\text{relative abundance of ureide} = \frac{(\text{ureide-N})}{(\text{ureide-N} + \text{amino acid-N} + \text{nitrate-N}) \times 100}
\]

**Assay for GS**

Nodule GS activity was assayed using the ADP-dependent transferase reaction (15) which measures the formation of GHA. One unit of GS activity is defined as 1 \mu mol of GHA formed per min. Two-hundred mg of fresh nodules were macerated in chilled mortars with 1.5 mL extraction buffer containing 40 mM imidazole-HCl (pH 7.2) and 10 mM MgCl\textsubscript{2}. Crude extracts were clarified by centrifugation in a microfuge for 6 min and the supernatant was used for the assays. All extraction procedures were done at 0 to 4°C.

**Ureide Degradative Capacity in Leaf Tissues**

Leaf ureide degradative capacity was assessed using an *in vivo* assay to measure conversion of allantoin to glyoxylate by a modification of the method reported by Schuller et al. (13). The assay medium contained 40 mM allantoin, 100 mM potassium phosphate (pH 7.5), and 1% (v/v) propanol. The leaf disc and assay media were evacuated for two-2 min intervals before incubation. After 1 h incubation, 1 mL aliquots was analyzed for glyoxylate according to Vogels and van der Drift (17).

**RESULTS**

Addition of nitrate to well nodulated soybean plants resulted in decreased nitrogenase activity (C\textsubscript{2}H\textsubscript{2} reduction) in all three of the nodulation mutants and in the wild-type Williams when assayed *in situ* (Fig. 1). The nitrogenase activity of the mutants was, however, much less affected than that of Williams; the latter was strongly inhibited by nitrate. Among the mutants tested, NOD1–3 showed the greatest nodulation tolerance to nitrate addition, regardless of whether C\textsubscript{2}H\textsubscript{2} reduction activity was expressed as a percentage of the respective nil nitrogen control (Fig. 1) or as an absolute activity value (data not shown).

![Figure 1. Effect of nitrate on *in situ* C\textsubscript{2}H\textsubscript{2} reduction activity of the nodulation mutants and Williams. See "Materials and Methods" for plant growth and assay method. The volume of nutrient solution was maintained at 5.5 L so that the majority of nodules were exposed to a 1.5 L gas phase starting 2 d before first assay and during subsequent growth and assay. Nitrate was not in direct contact with nodules which were assayed by C\textsubscript{2}H\textsubscript{2} reduction. On d 0, C\textsubscript{2}H\textsubscript{2} reduction activity for Williams, NOD1–3, NOD2–4, and NOD3–7 were 21.9, 19.8, 20.3, and 23.1 \mu mol plant\textsuperscript{-1} h\textsuperscript{-1}, respectively. The C\textsubscript{2}H\textsubscript{2} reduction activity was measured each day, and treatments were expressed as percent of their own control for that day. Three replicates (four plants per replicate) were assayed. The vertical bar represents the largest so within any given sample time.](image-url)
Because 3-week-old plants did not yield sufficient root-bleeding sap for analysis, dry tissue extracts were used to determine relative abundance of ureides in plant parts to assess the effect of nitrate on the products of nitrogen fixation. The stems (including petioles) and leaves were used for ureide, amino acid, and nitrate assays. Stems have previously been shown to be a more suitable indicator of N\(_2\) fixation capacity, based on the relative abundance of ureide (7). Figure 2 shows the changes in relative abundance of ureides in stems as affected by addition of 5 mM nitrate. The decreases in stem tissue relative ureide abundance (Fig. 2) were similar to the declines in nitrogenase activity determined by the C\(_2\)H\(_2\) reduction assay (Fig. 1). The relative abundance of ureide data confirmed that the selected nodulation mutants were less susceptible to nitrate inhibition of nitrogenase activity compared with Williams; the latter also exhibited the greatest decline in C\(_2\)H\(_2\) reduction activity upon nitrate treatment. The NOD1–3 line also had a higher relative abundance of ureides in stems in the absence of nitrate (inset, Fig. 2).

Relative abundance of ureides in leaves showed a distinctly different pattern between the nodulation mutants and the Williams parent (Fig. 3). Nitrate treatment resulted in an initial increase in the relative abundance of ureides in leaves of the nodulation mutants and a decrease in relative abundance of ureide in leaves of Williams. It was also observed that actual ureide concentration increased in each plant part tested following 2 d of nitrate treatment (Table I). Similar responses were noted in the NOD2–4 and NOD3–7 mutants (data not shown).

Because NOD1–3 showed the greatest difference, when

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**Figure 2.** Effect of nitrate on relative abundance of ureides in stems of the nodulation mutants and Williams. Relative abundance of ureides is defined as ureide-N divided by the sum of ureide-N, amino acid-N, and nitrate-N (see "Materials and Methods"). Inset represents control without nitrate treatment. Other details as in Figure 1 legend, except that plants were transferred into a nil nitrate solution on d 4.

**Figure 3.** Effect of nitrate on relative abundance of ureides in leaves of the nodulation mutants and Williams. Other details as in Figure 2 legend.

**Table I.** Effect of 5 mM Nitrate Addition following the d 0 Sampling on Root (Including Nodules), Stem, and Leaf Ureide Concentrations

<table>
<thead>
<tr>
<th>Soybean Lines</th>
<th>Ureide Concentrations</th>
<th>Day 0</th>
<th>Day 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant Parts</td>
<td>(\mu\text{mol ureides mg}^{-1}\text{ dry weight})</td>
<td></td>
<td></td>
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<tr>
<td>Nod 1-3</td>
<td>Roots</td>
<td>0.30 ± 0.02</td>
<td>0.35 ± 0.09 (+17)</td>
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<tr>
<td></td>
<td>Stems</td>
<td>1.20 ± 0.11</td>
<td>1.51 ± 0.24 (+26)</td>
</tr>
<tr>
<td></td>
<td>Leaves</td>
<td>0.60 ± 0.06</td>
<td>0.70 ± 0.03 (+17)</td>
</tr>
<tr>
<td></td>
<td>Williams</td>
<td>0.29 ± 0.01</td>
<td>0.38 ± 0.04 (+31)</td>
</tr>
<tr>
<td></td>
<td>Roots</td>
<td>0.66 ± 0.08</td>
<td>1.05 ± 0.04 (+59)</td>
</tr>
<tr>
<td></td>
<td>Leaves</td>
<td>0.47 ± 0.01</td>
<td>0.62 ± 0.08 (+32)</td>
</tr>
</tbody>
</table>
SOYBEAN NODULATION MUTANTS

correlated to Williams, it was evaluated in more detail. Nodules were removed and analyses of specific nodule activity and ureide level were performed. The detailed study of NOD1-3 and Williams confirmed that this nodulation mutant retained higher C$_2$H$_2$ reduction activity upon nitrate addition (56% versus 41% of respective controls) (Table II). The nodule fresh weight of NOD1-3 was 1.8- and 2.1-fold greater than Williams in the absence and presence of nitrate, respectively. The difference in nodule fresh weight between lines did not reflect any nitrate treatment effect because 2 d of nitrate treatment was insufficient to have any marked effect on nodule mass. The partial tolerance to nitrate was apparently associated with the hypernodulated phenotype. Although per plant C$_2$H$_2$ reduction activity in the presence of nitrate was higher for the NOD1-3 mutant than for Williams, the activity per g of nodule fresh weight was lower for NOD1-3 than for Williams, both in the absence and presence of nitrate (Table II).

There were no differences in GS activity of nodules between NOD1-3 and Williams, nor between control and nitrate-treated plants (Table III). This indicates that NOD1-3 and Williams have similar capability of assimilating N that is symbiotically fixed. There were only slight differences in leaf ureide degradative capacity between the NOD1-3 mutant and Williams when analyzed before nitrate treatment. Leaf ureide degradative capacity, however, increased upon nitrate application (Table III) as did leaf ureide levels (Table I). It did not appear that ureide degradative capacity was directly linked to ureide level since both NOD1-3 and NOD3-7 lines had similar ureide degradative capacity and yet NOD3-7 accumulated two times as much ureide as did NOD1-3 (data not shown).

**DISCUSSION**

Availability of soil N is likely the major environmental limitation to nodulation of soybean. Having a soybean line which would nodulate and symbiotically fix N$_2$ in the presence of nitrate would allow field testing of whether (a) overall nitrogen metabolism can be enhanced, and (b) this would have a positive impact on growth and yield. The selection of soybean mutants in which nodulation is partially tolerant of nitrate (1, 2, 4) has provided new insight into the control of nodulation and may allow increased N$_2$ fixation in soils which have high residual levels of soil N. The major difference in the mutants involves alteration of the autoregulation control of nodulation (1), although partial nodulation tolerance to nitrate also exists (4, 13). The question remains as to whether these selected mutants have sufficient tolerance to nitrate to realize any gain in overall nitrogen metabolism. The current study was conducted to enable separation of nitrate effects on nodule function (nitrogenase activity) per se from effects of nitrate on the infection/initial nodule development phase.

Gremaud and Harper (4) reported that nitrate was less inhibitory to infection and initial nodule development in the NOD1-3, NOD2-4, and NOD3-7 mutant lines than in the Williams wild-type line. It has also been confirmed by $^{15}$N analyses that the three selected mutants are capable of symbiotically fixing more N$_2$ than does the Williams parent when plants were grown on nitrate (11). Both studies, however, involved simultaneous inoculation and nitrate application which prevented separation of possible nitrate effects on the infection stage and the functional activity (nitrogenase) stage of nodulation. The current study extends that observation to an evaluation of whether nitrate is detrimental to functional activity of preexisting nodules, as assessed by C$_2$H$_2$ reduction assays and the relative abundance of ureide technique. Plants were allowed to undergo normal nodule development in the absence of nitrate and to then determine the short-term impact of nitrate addition on nodule function.

The overall patterns of decline in C$_2$H$_2$ reduction activity (Fig. 1) and relative ureide abundance in stems (Fig. 2) indicated that both assays would lead to similar conclusions concerning the short-term effect of nitrate on nitrogenase activity. Acetylene reduction activity did, however, decline more rapidly with time after nitrate addition than did the relative abundance of ureides. This was expected because ureide pools may exist in nodules which could be translocated even after nitrogenase activity has been inhibited. Second, it has been observed that the field-grown soybean nonnodulated lines (our unpublished data) or the field-grown lines in which

<table>
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<tr>
<th>Parameters</th>
<th>NOD1-3 Control</th>
<th>NOD1-3 5 mm NO$_3^-$</th>
<th>Williams Control</th>
<th>Williams 5 mm NO$_3^-$</th>
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<tr>
<td>Nodule fresh wt (g plant$^{-1}$)</td>
<td>1.57 ± 0.07</td>
<td>1.62 ± 0.13 (+7)</td>
<td>0.88 ± 0.02</td>
<td>0.78 ± 0.08 (-11)</td>
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<td>C$_2$H$_2$ reduction Per plant</td>
<td>28.9 ± 3.11</td>
<td>16.3 ± 2.31 (-44)</td>
<td>25.3 ± 2.74</td>
<td>10.2 ± 0.58 (-59)</td>
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<tr>
<td>Per g nodule fresh wt</td>
<td>18.4 ± 1.17</td>
<td>10.1 ± 0.64 (-47)</td>
<td>28.7 ± 2.43</td>
<td>13.1 ± 0.69 (-54)</td>
</tr>
<tr>
<td>Nodule N Ureide</td>
<td>0.56 ± 0.01</td>
<td>0.80 ± 0.11 (+43)</td>
<td>0.48 ± 0.03</td>
<td>0.64 ± 0.08 (+33)</td>
</tr>
<tr>
<td>Amino acid</td>
<td>2.63 ± 0.04</td>
<td>3.02 ± 0.06 (+15)</td>
<td>2.19 ± 0.18</td>
<td>2.12 ± 0.11 (-3)</td>
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<tr>
<td>Nitrate</td>
<td>0.00</td>
<td>1.36 ± 0.08</td>
<td>0.00</td>
<td>0.93 ± 0.24</td>
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nodulation was completely inhibited by nitrate (8) still have 10 to 20% relative abundance of ureide in vacuum-extracted xylem sap. This indicates that C2H2 reduction activities will be more sensitive to changes in nitrogenase activity than will the ureide abundance technique. This conclusion was supported by the data in Figures 1 and 2 where C2H2 reduction activity for Williams declined to about 5% of control whereas the relative abundance of ureides in stems stabilized at about 18%.

The observation that C2H2 reduction activity of the NOD1-3 line was less affected by nitrate than was Williams (Fig. 1) was only partially supported by a higher relative ureide abundance in the stems of NOD1-3 (Fig. 2). Although on d 2 and 4 following nitrate addition the NOD1-3 line retained significantly higher relative abundance of stem ureides than did Williams, NOD1-3 was not significantly different from the other two mutants. In the absence of nitrate, the relative abundance of ureides in stems was consistently higher in the NOD1-3 line (Fig. 2, inset), leading to the conclusion that this mutant was more capable of N2 fixation than the other two mutants or Williams in the absence of nitrate. A higher relative abundance of ureides in leaves of the NOD1-3 line was also noted (Fig. 3, inset) which is consistent with a nodulation advantage of this line. Likewise, the C2H2 reduction activity on a per plant basis was higher for NOD1-3 than for Williams, both in the absence and presence of nitrate (Table II). It must be noted, however, that C2H2 reduction activity on a unit nodule basis was less for the NOD1-3 line than for Williams (Table II), which is similar to the observations of Schuller et al. (13) with their nts382 supernodulation line.

The higher rates of C2H2 reduction activity measured in vivo (Table II) than measured in situ (Fig. 1, legend) were likely due to the fact that the in situ measurements were confined to the upper nodulated root segments exposed in the 1.5-L gas space, whereas the entire nodulated root system was exposed during the in vivo assay. Certainly there were nodules which were submersed in the remaining nutrient solution during the in situ assay. This was more pronounced with the nodulation mutants than with Williams, the latter having more nodules confined to the upper tap root region. Although the validity of the closed system C2H2 reduction assay has been questioned due to possible changes in the oxygen diffusion barrier (10), we have verified that C2H2 production is linear through a 30 min assay period with our culture and assay system (our unpublished data).

Our results (Table I and II) confirmed observations of Yoneyama et al. (19) that nitrate treatment resulted in accumulation of ureide in essentially every plant part. The increase of ureide concentration in response to short-term nitrate treatment was, however, overshadowed by nitrate uptake and accumulation in plant parts such that the relative abundance of ureide in stems still decreased in nitrate-treated plants (Fig. 2). An unexpected result of the present study was that, in addition to accumulation of ureide in leaves in response to nitrate, the leaves also showed a parallel increase in ureide degradative capacity (Table III). It is thought that ureides accumulate in tissues due to preferential use of nitrate, but this does not explain why greater ureide degradative capacity was also observed. This area deserves further investigation.

The independent selection of nodulation mutants from two diverse backgrounds of soybean, Bragg (1, 2) and Williams (4), in which different mutagenic treatments were used, has indicated that gene control for this trait is relatively unstable. Naturally occurring lines which exhibit the nts phenotype described by Gresshoff's laboratory or the excess nodulation described for lines from our laboratory have not, however, been identified. This is surprising given the relative frequency of observed mutants obtained experimentally and the strong nitrate selection pressure which Midwest soybean plants are subjected to. Estimates indicate that some 50 to 75% of the total plant N is derived from the soil (5). This soil N is primarily nitrate and hence should exert considerable natural selection pressure for variants which would nodulate in the presence of nitrate. Lack of these naturally occurring variants may indicate that N metabolism is not limiting to soybean production under typical management conditions and, hence, there may not be natural pressure to select for lines which nodulate in the presence of nitrate. In view of this, it may prove that the selected nodulation mutants will not have any agronomic advantage. The current importance of these mutants may then be to allow assessment of possible control points of the nodulations process.

ACKNOWLEDGMENT

We thank Joe Nichols for his expert assistance.

LITERATURE CITED


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<td>μmol glyoxylate g⁻¹ leaf fresh wt h⁻¹</td>
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<td>54.4 ± 10.7</td>
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<td>5 mM NO₃⁻</td>
<td>55.7 ± 8.9</td>
<td>0.21 ± 0.04</td>
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<td>Williams</td>
<td>Control</td>
<td>46.9 ± 3.8</td>
<td>0.04 ± 0.00</td>
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<td></td>
<td>5 mM NO₃⁻</td>
<td>45.5 ± 5.7</td>
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and nitrate-tolerant symbiotic (nts) soybean mutant. Plant Physiol 78: 34-40